Use of *CBL* exon 8 and 9 mutations in diagnosis of myeloproliferative neoplasms and myelodysplastic/myeloproliferative disorders: an analysis of 636 cases

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ABSTRACT

We analyzed 636 patients with diverse myeloproliferative neoplasms or myelodysplastic/myeloproliferative neoplasms for mutations of the Casitas B-cell lymphoma gene (*CBL*^{mut}) in exons 8 and 9 and performed correlations to other genetic alterations. *CBL*^{mut} were detected in 63 of 636 (9.9%) of these selected patients. *CBL*^{mut} were more frequent in myelodysplastic/myeloproliferative neoplasms than myeloproliferative neoplasms (51 of 328, 15.5% *vs.* 12 of 291, 4.1%; $P<0.001$). Frequency was 48 of 278 (17.3%) in chronic myelomonocytic leukemia and 3 of 33 (9.1%) in unclassifiable myelodysplastic/myeloproliferative neoplasms. *CBL*^{mut} was not detected in polycythemia vera, primary myelofibrosis, essential thrombocythemia, or refractory anemia with ring sideroblasts and marked thrombocytosis. *CBL*^{mut} were underrepresented in *JAK2*^{V617F} mutated as compared to *JAK2*V617^{wt} cases ($P<0.001$), and mutually exclusive of *JAK2*exon12^{mut} and *MPL*W515^{mut}. *CBL*^{mut} were associated with monosomy 7 ($P=0.008$) and *TET2*^{mut} ($P=0.003$). In chronic myelomonocytic leukemia, *CBL*^{mut} had no significant impact on survival outcomes. Therefore, *CBL*^{mut} are frequent in chronic myelomonocytic leukemia, absent in classical myeloproliferative neoplasms, and are only exceptionally found in coincidence with JAK-STAT pathway activating mutations.

Key words: *CBL* mutations, chronic myelomonocytic leukemia, myeloproliferative neoplasms, prognosis, sequencing.

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Introduction

The Casitas B-cell lymphoma gene (*CBL*) (on chromosome 11q23.3) contains several functional domains. One of these, the C-terminal domain, gives rise to the ubiquitin activity site of the Cbl protein. By ubiquitination, the Cbl protein is targeting multiple sites of receptor tyrosine kinases, e.g. PDGFR or FLT3, resulting in negative modulation of tyrosine kinase signaling.[1] Mutations in *CBL* (*CBL*^{mut}) were first identified due to acquired uniparental disomy (UPD) of 11q in myeloid neoplasms.[1-3] These mutations lead to dysregulation of receptor tyrosine kinases and have the potential to transform hematopoietic cells by constitutively activating the FLT3 pathway.[4] With regards to the myeloid entities which can be affected by these mutations, Dunbar *et al.* identified *CBL*^{mut} in 7 of 12 patients with uniparental disomy (UPD) of 11q in a cohort of 301 patients with different myeloid disorders including MDS, the MDS/MPN overlap category, MPNs, and acute myeloid leukemia (AML).[2] Grand *et al.* found *CBL*^{mut} in 8% of atypical chronic myeloid leukemia (aCML), 6% of myelofibrosis, and 1% of hypereosinophilic syndrome/chronic eosinophilic leukemia (HES/CEL) cases.[3] Beer *et al.* documented a patient in whom a *CBL*^{mut} was detectable in megakaryocytes two years before transformation from MPN to AML.[5] Very heterogeneous frequencies of *CBL*^{mut} were reported in chronic myelomonocytic leukemia (CMML) ranging from 5%[6] to 22%.[7] Detailed analysis in other entities has been scarce. To evaluate the role of *CBL*^{mut} in diverse MPNs and myelodysplastic/myeloproliferative neoplasms (MDS/MPN), we analyzed *CBL*^{mut} in a large cohort of 636 adult patients and performed correlation studies with other molecular mutations, karyotypes, and clinical outcomes.

Design and Methods

The study cohort was made up of 636 patients: 291 patients had MPNs (polycythemia vera, PV, n=32; essential thrombocythemia, ET, n=48; primary myelofibrosis, PMF, n=19; unclassifiable MPN, n=175; so-called 'advanced MPN' (corresponding to an accelerated phase of an MPN or s-AML following a previous MPN n=17). A total of 328

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patients had disorders from the WHO overlap category of myelodysplastic/myeloproliferative neoplasms (CMML-1, n=194; CMML-2, n=84; unclassified MDS/MPN, n=33; RARS-T, n=17),[8] and 17 patients had HES/CEL. Details of some of the CMML[7] (81 of 278) and RARS-T[9] patients have been published previously and 4 CMML cases have recently been published elsewhere for clinical and histological analysis.[10] Demographic data and blood values are shown in Table 1. Diagnoses were performed according to the WHO.[8] There were 389 males and 247 females (male/female ratio 1.6) with a median age of 70.7 years (range 18.4-93.3). Patients were selected according to the availability of cytomorphology, cytogenetics and molecular genetic characterization. Samples were referred to the MLL Munich Leukemia Laboratory in the period from August 2005 to April 2011. Patients gave their written consent. The study was approved by the Internal Review Board of the Munich Leukemia Laboratory in accordance with the Declaration of Helsinki.

Bone marrow and/or peripheral blood samples underwent May Grünwald Giemsa staining and cytochemistry with myeloperoxidase (MPO) and non-specific esterase (NSE).[11] Chromosome banding analysis was carried out in all 636 cases, combined with fluorescence *in situ* hybridization (FISH) when necessary.[12] Patients were assigned to the following cytogenetic subgroups: normal karyotype, -Y (in male patients), gain of 1q, chromosome 7 abnormalities, trisomy 8 as sole abnormality, 12p deletion, 20q deletion, complex karyotype (defined by ≥3 chromosomal abnormalities), reciprocal translocations, other trisomies, and other alterations (*Online Supplementary Table S1*).

CBL^{mut} analysis was performed by direct Sanger sequencing covering exons 8-9.[3] Mutation loads were estimated visually from electropherograms of forward and reverse reactions as generated by Sanger sequencing and confirmed by pyrosequencing[7] in half of the cases with good correlations. In addition, *BCR-ABL1* was excluded by multiplex RT (reverse transcription)-PCR in all patients.[13] Mutation analysis was carried out in subsets of patients: *JAK2*V617F (n=635),[14] *JAK2* exon 12 mutations (n=632),[15] *MPL* (n=634),[16] *RUNX1* (n=305),[17] *EZH2* (n=279),[18] *TET2* (n=320),[7] *NRAS* (n=312),[19] *KRAS* (n=294),[7] and *ASXL1* (n=271; by direct Sanger sequencing of exon 12).[20]

Overall survival was the interval from the first evaluation of the patient´s sample in the Munich Leukemia Laboratory to death. Median overall survival (OS) was calculated according to Kaplan Meier and compared by two-sided log rank test. Dichotomous variables were compared by the χ^2 test, continuous variables by Student´s t-test. SPSS (version 19.0.0, IBM, Ehningen, Germany) software was used for statistical analysis.

Results and Discussion

In the total cohort, *CBL*^{mut} were detected in 63 of 636 (9.9%) patients. Localization of the mutations in the LINKER and RING domain (exons 8-9) is shown in Figure 1A. When the different diagnostic entities were compared, *CBL*^{mut} were more frequent in MDS/MPN than in MPN (51 of 328, 15.5% *vs.* 12 of 291, 4.1%; P<0.001). In MDS/MPN, the frequency of *CBL*^{mut} was highest in CMML with 48 of 278 (17.3%) of all cases (CMML-1: 36 of 194, 18.6%; CMML-2: 12 of 84, 14.3%) being followed by MDS/MPNu (3 of 33, 9.1%). No *CBL*^{mut} was identified in the 17 RARS-T patients. Therefore, the high frequency in MDS/MPN was due to the overrepresentation in CMML. Within the MPN category, the frequency was highest in MPNu (11 of 175, 6.4%) and advanced MPN (1 of 17, 5.9%). No *CBL*^{mut} was identified in PV (n=32), PMF (n=19), ET (n=48) or HES/CEL (n=17). Taken together, due to their high frequency in CMML, *CBL*^{mut} showed a higher frequency in the overlap MDS/MPN category as compared to the MPN category, and were not detected within clearly defined entities such as ET, PV, PMF, HES/CEL, or RARS-T.

CBL^{mut} were strongly underrepresented in *JAK2*^{V617F} mutated as compared to *JAK2*V617^{wt} patients (1 of 121, 0.8% *vs.* 62 of 514, 12.1%; *P*<0.001). *CBL*^{mut} was detected concomitantly with *JAK2*^{V617F} in only one case. This case showed a high load of *CBL*^{mut} alleles in contrast to a low mutation *JAK2*^{V617F} level of 1%. *CBL*^{mut} were mutually exclusive of *JAK2*exon12 (n=6) and *MPL* (n=13) mutations. The frequency of *CBL*^{mut} was lower in *NRAS*^{mut} as compared to *NRAS*^{wt} cases (2 of 45, 3.6% *vs.* 54 of 267, 20.2%; *P*=0.010) and *KRAS*^{mut} as compared to *KRAS*^{wt} cases (1 of 29, 3.4% *vs.* 49 of 265, 18.5%; *P*=0.038). In con-

Table 1. *CBL*^{mut} frequency, demographic data, and peripheral blood values in the different entities.

Category	N. of cases	No. of *CBL*^{mut} (frequency)	Male/female (ratio)	Median age (range), years	Median peripheral blood values (range) WBC, x10^9/L	Hb, g/dL	Thrombocytes, x10^9/L
Total cohort	**636**	**63 (9.9%)**	**389/247 (1.6)**	**70.7 (18.4-93.3)**	**12.9 (0.9-729.9)**	**11.9 (4.0-22.5)**	**220 (3-3,528)**
MPN category	**291**	**12 (4.1%)**	**160/131 (1.2)**	**67.0 (22.8-90.1)**	**15.0 (0.9-140.0)**	**12.9 (4.8-22.5)**	**484 (10-3,528)**
PV	32	0 (0.0%)	22/10 (2.2)	65.9 (36.8-84.6)	11.6 (4.9-31.6)	18.1 (9.1-22.5)	449 (200-1,776)
PMF	19	0 (0.0%)	10/9 (1.1)	67.2 (54.7-87.6)	6.9 (2.5-62.0)	10.0 (5.6-13.3)	184 (40-418)
ET	48	0 (0.0%)	19/9 (0.7)	64.7 (22.8-80.3)	8.8 (4.0-12.0)	13.6 (7.1-18.8)	940 (478-2,095)
MPNu	175	11 (6.4%)	100/75 (1.3)	68.3 (23.0-90.1)	17.7 (2.0-729.9)	12.7 (4.8-19.9)	420 (10-3,528)
Advanced phase MPN	17	1 (5.9%)	9/8 (1.1)	69.1 (47.7-88.0)	22.2 (2.4-164.6)	10.1 (5.6-13.3)	256 (29-1,027)
MDS/MPN category	**328**	**51 (15.5%)**	**219/109 (2.0)**	**73.1 (21.9-93.3)**	**15.0 (0.9-140.0)**	**10.7 (4.0-19.7)**	**95 (3-1,500)**
CMML-1	194	36 (18.6%)	140/54 (2.6)	73.3 (21.9-90.5)	16.4 (1.6-129.2)	11.0 (5.9-18.2)	92 (3-1,385)
CMML-2	84	12 (14.3%)	54/30 (1.8)	71.6 (29.7-93.3)	11.7 (0.9-113.2)	11.0 (4.0-15.8)	86 (21-711)
MDS/MPNu	33	3 (9.1%)	20/13 (1.5)	74.3 (45.3-88.4)	18.7 (2.9-140.0)	10.1 (6.2-19.7)	104 (20-757)
RARS-T	17	0 (0.0%)	5/12 (0.4)	75.9 (59.0-79.8)	6.5 (4.6-60.0)	9.8 (4.0-22.5)	693 (466-1,500)
HES/CEL	**17**	**0 (0.0%)**	**10/7 (1.4)**	**51.6 (18.4-79.8)**	**10.5 (2.7-71.4)**	**14.0 (10.0-16.9)**	**286 (35-637)**

WBC: white blood cells; Hb: hemoglobin.

Table 2. Frequency of *CBL*^{mut} in different molecular subgroups (*P* values were calculated by χ^2 test). *CBL*^{mut} rates are given within the molecular subgroups as defined by *JAK2V617F*, *JAK2*exon12, *MPLW515*, *RUNX1*, *ASXL1*, *EZH2*, *TET2*, and *NRAS* mutation status.

Molecular mutation (n=mutated/cases analyzed)	Mutation status*	*CBL*^{mut} (%)	*P*
*JAK2*V617F (n=121/635; 19.1%)	+	1/121 (0.8)	<0.001
	-	62/514 (12.1)	
*JAK2*exon12 (n=6/632; 0.9%)	+	0/6 (0.0)	n.s.
	-	63/626 (10.1)	
*MPLW*515 (n=13/634; 2.1%)	+	0/13 (0.0)	n.s.
	-	63/621 (10.1)	
RUNX1 (n=67/305; 22.0%)	+	13/67 (19.4)	n.s.
	-	43/238 (18.1)	
ASXL1 (n=122/271; 45.0%)	+	23/122 (18.9)	n.s.
	-	21/149 (14.1)	
EZH2 (n=28/279; 14.3%)	+	4/28 (14.3)	n.s.
	-	35/251 (13.9)	
TET2 (n=25/320; 7.8%)	+	25/135 (18.5)	0.003
	-	13/185 (7.0)	
NRAS (n=45/312; 14.4%)	+	2/45 (3.6)	0.010
		54/267 (20.2)	
KRAS (n=29/294; 9.9%)	+	1/29 (3.4)	0.038
	-	49/265 (18.5)	

* + mutated; - wild-type.

trast, *CBL*^{mut} showed a significantly higher frequency in *TET2*^{mut} cases as compared to *TET2*^{wt} (25 of 135, 18.5% *vs.* 13 of 185, 7.0%; *P*=0.003) (Table 2; Figure 1B). There was no significant difference in *CBL*^{mut} dependence on the *RUNX1*, *ASXL1*, and *EZH2* mutation status (Table 2).

Summarizing these results, presence of *CBL*^{mut} with the *JAK2*^{V617F} was extremely rare, and *CBL*^{mut} seem to show mutual exclusiveness of *JAK2*exon12 and *MPL*W515 mutations. This gives rise to the hypothesis that *CBL*^{mut} do not play a role in the 'classical' MPNs, although larger numbers of patients and the whole *CBL* gene would have to be analyzed for definite conclusions to be drawn. Furthermore, *CBL*^{mut} were significantly underrepresented in *NRAS*^{mut} (*P*=0.010) and *KRAS*^{mut} (*P*=0.038) patients in our study. Also in pediatric JMML, no *CBL*^{mut} case was detected in 91 patients with RAS pathway activating mutations (*P*<0.001)[21] and a single double mutated case only was identified in CMML.[21] Therefore, *CBL*^{mut} and JAK-STAT activating mutations largely seem to exclude each other, although, here again, larger numbers of patients and the whole *CBL* gene would have to be analyzed for definite conclusions to be drawn. This is in accordance with the function of *CBL*, as it is involved in negative modulation of tyrosine kinase signaling, and, therefore, does itself finally end up in the JAK-STAT pathway. *CBL*^{mut} in addition to another JAK-STAT activating mutation would probably not result in a further growth advantage for the respective cell. In contrast, Aranaz *et al.* found the same frequencies of *CBL*^{mut} in patients with *JAK2*V617F-positive and -negative MPNs; however, these were only a very few cases each.[22] This suggests that such a coincidence still might occur very rarely, and it is still debatable as to whether in these rare cases two different subclones coexist. As we found a *CBL*^{mut} rate of 5.9% in advanced MPNs in our study, it may be speculated that the respective mutations may contribute to disease progression in the MPNs, which is also in accordance with data on blast phase of chronic myeloid leukemia.[23,24]

Correlation of *CBL*^{mut} with different cytogenetic subgroups (*Online Supplementary Table S1*) revealed the highest frequency in patients with monosomy 7. *CBL*^{mut} were more frequent in patients with monosomy 7 (4 of 9, 44.4%) when compared to all remaining cases (59 of 627, 9.4%; *P*=0.008). *CBL*^{mut} showed no significant correlations with other frequent cytogenetic subgroups, i.e. normal karyotypes, trisomy 8, or loss of Y chromosome.

Of all 63 *CBL*^{mut} patients, 56 (88.9%) had only one *CBL*^{mut}. Of these 56, 37 had a mutation/wild-type load of 50% or less and 19 had a load of over 50%. Eight (12.7%) cases had two different *CBL*^{mut} in parallel. These cases were reanalyzed by pyrosequencing for better quantification of the mutation load, which in all cases was more than 50%. Combination of mutations and load were as follows: 1) p.Ile423Asn (38%) + p.Val430Met (40%); 2) p.Cys404Tyr (31%) + p.Arg420Gln (36%); 3) p.Cys384Arg (84%) + p.Met400Arg (7%); 4) p.His398Arg (90%) + p.Ile429_Phe434del (6%); 5) p.Cys416Ser (43%) + p.Arg420Gly (38%); 6) p.Arg420Gln (15%) + p.Arg420X (72%); 7) p.Gly415Ser (38%) + p.Arg462X (42%); 8) p.Asp390Tyr (40%) + splicing of exon 9 (31%). In all 4 cases in whom the mutations were located on the same amplicon, they were shown to appear on different alleles. Based on these data, it was not possible to draw definite conclusions as to whether these mutations were in different clones or whether both alleles of one clone were mutated. The mean mutation load in all patients was 55.0±26.0%. There was no significant difference in mean mutation load between CMML patients and the other *CBL*^{mut} patients (59.0±29.1% *vs.* 53.1±24.4%; n.s.).

Most (n=57, 90.5%) alterations were missense mutations. Three cases had small deletions (p.Tyr368_Glu369del; p.Leu370_Tyr371del; and p.Ile429_Phe434del), 2 further cases revealed a stop mutation (p.Arg420X, and p.Arg462X), and one case an exon 9 splice mutation. Some mutations were recurrent in our cohort, such as p.Arg420Gln (n=4), p.Phe418Ser (n=3), p.Arg420Leu (n=2), p.Cys404Tyr (n=2), p.Cys416Arg (n=2), p.Ile383Met (n=2), p.Ile429Asn (n=2), and p.Leu380Pro (n=2), whereas all others were detected in single cases only (Figure 1A).

Biological characteristics and peripheral blood values were compared between *CBL*^{mut} and *CBL* wild-type (*CBL*^{wt}) cases in the CMML cohort (n=278). The male/female ratio was higher in the *CBL*^{mut} CMML patients than in *CBL*^{wt} patients (5.0 *vs.* 2.0; *P*=0.025). No significant differences were found regarding median age or peripheral blood parameters between *CBL*^{mut} and *CBL*^{wt} cases in CMML (*Online Supplementary Table S2*).

Because of the high prevalence of *CBL*^{mut} in CMML, outcome analysis was performed only in this subcohort. Clinical follow-up data were available in 176 of 278 CMML patients (36 *CBL*^{mut}, 140 *CBL*^{wt}). Median overall survival (OS) of the whole CMML cohort was 29.9 months (CMML-1: median OS not reached; CMML-2: median OS 29.6 months; n.s.). Within the whole CMML cohort, there was no significant difference between OS of patients with *CBL*^{mut} and that of those with *CBL*^{wt} (median 32.4 *vs.* 29.9 months). When the CMML-1 cohort (follow-up data available in 112 patients) was investigated separately, *CBL*^{mut} patients had shorter OS than *CBL*^{wt} (median

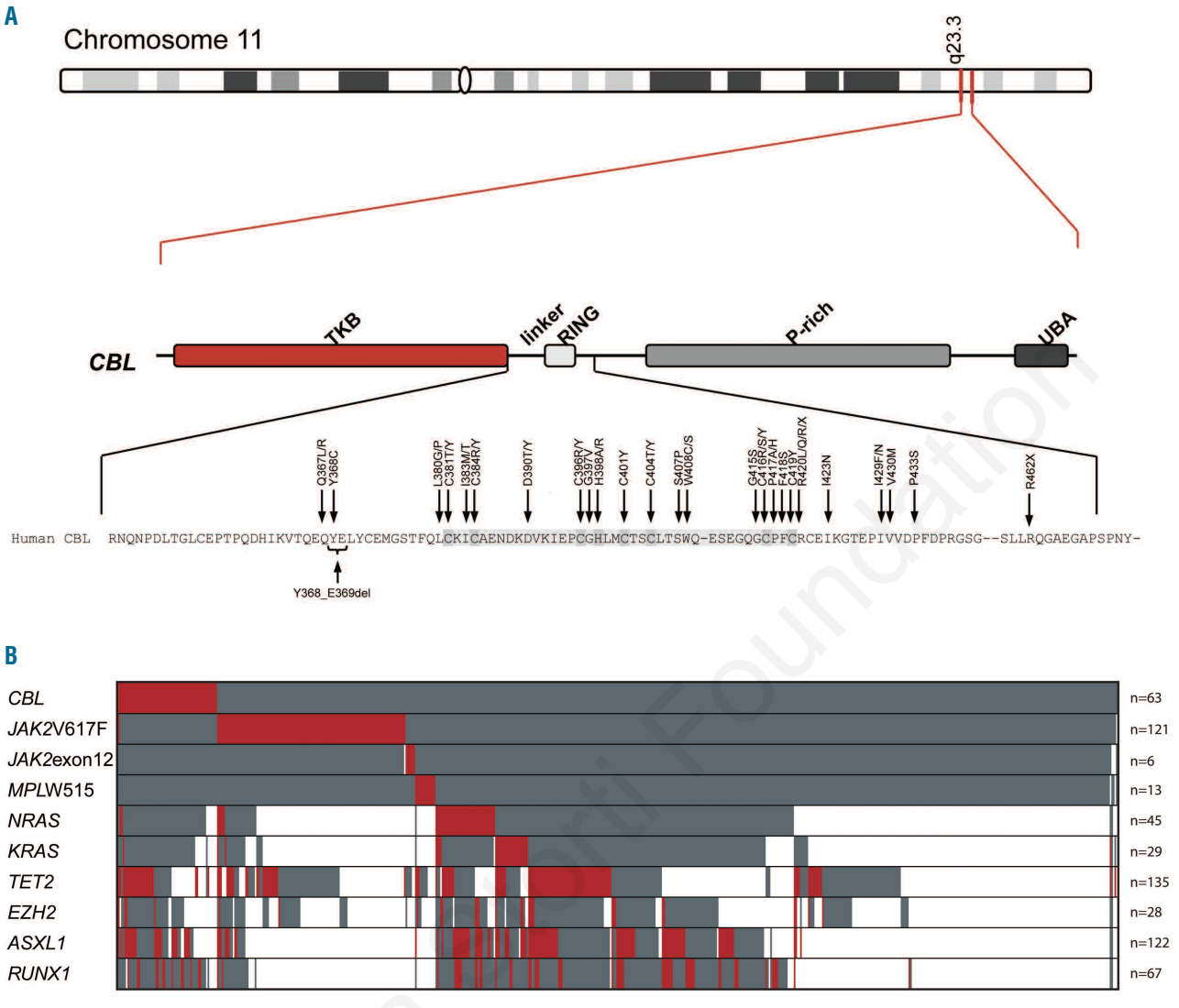

Figure 1. (A) Illustration of the localization of the different mutations within the *CBL* gene and of the corresponding amino acids detected in the patients of this study. The highest conserved domains are marked in dark gray. Top position of *CBL* at chromosomal band 11q23.3. Middle panel: structure of the gene according to Grand *et al.*[3] with TKB (tyrosine kinase binding) domain, linker and Ring domain, P-rich (Proline rich) domain and UBA (ubiquitin-associated) domain. Bottom amino acid exchanges detected in our cohort. (B) Distribution and frequency of CBL^{mut} and other molecular mutations in the total cohort of 636 patients. Red indicates a mutation within the respective gene, gray indicates no mutation. White cells indicate that the respective gene mutation was not analyzed for this patient. Patients are presented vertically.

25.4 months *vs.* median not reached; *P*=0.227), but this difference did not reach significance. In the CMML-2 cohort (n=64 patients with survival data), survival outcomes were very similar between CBL^{mut} and CBL^{wt} patients (32.4 *vs.* 24.8 months; n.s.; *Online Supplementary Figure S1*). Corresponding to our previous analysis, including some of the patients from this study,[7] patients with CBL^{mut} had shorter OS when compared to those with CBL^{wt} in the CMML-1 cohort; but this difference did not reach significance. Therefore, the prognostic value of CBL^{mut} in CMML and in the MPNs, and its contribution to disease progression, deserves further investigation.[25]

In conclusion, CBL^{mut} are overrepresented in CMML when compared to the MPNs. They rarely occur together with JAK-STAT pathway activating mutations, but are frequently seen with other genetic markers, e.g. mutations of the *TET2* gene. Because of the high frequency for CMML and certain exclusion patterns with other mutations, CBL^{mut} analysis is a useful additive tool for differential diagnosis.

Authorship and Disclosures

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